

EXHIBIT A

BEST AVAILABLE COPY

Synergistic Antitumor Activity of Histamine Plus Melphalan in Isolated Limb Perfusion: Preclinical Studies

Flavia Brunstein, Saske Hoving, Ann L. B. Seynhaeve, Sandra T. van Tiel, Gunther Guetens, Ernst A. de Bruijn, Alexander M. M. Eggermont, Timo L. M. ten Hagen

Background: We have previously shown how tumor response of isolated limb perfusion (ILP) with melphalan was improved when tumor necrosis factor alpha (TNF- α) was added. Taking into account that other vasoactive drugs could also improve tumor response to ILP, we evaluated histamine (Hi) as an alternative to TNF- α . **Methods:** We used a rat ILP model to assess the combined effects of Hi and melphalan ($n = 6$) on tumor regression, melphalan uptake ($n = 6$), and tissue histology ($n = 2$) compared with Hi or melphalan alone. We also evaluated the growth of BN-175 tumor cells as well as apoptosis, necrosis, cell morphology, and paracellular permeability of human umbilical vein endothelial cells (HUVECs) after Hi treatment alone and in combination with melphalan. **Results:** The antitumor effect of the combination of Hi and melphalan *in vivo* was synergistic, and Hi-dependent reduction in tumor volume was blocked by H_1 and H_2 receptor inhibitors. Tumor regression was observed in 66% of the animals treated with Hi and melphalan, compared with 17% after treatment with Hi or melphalan alone. Tumor melphalan uptake increased and vascular integrity in the surrounding tissue was reduced after ILP treatment with Hi and melphalan compared with melphalan alone. *In vitro* results paralleled *in vivo* results. BN-175 tumor cells were more sensitive to the cytotoxicity of combined treatment than HUVECs, and Hi treatment increased the permeability of HUVECs. **Conclusions:** Hi in combination with melphalan in ILP improved response to that of melphalan alone through direct and indirect mechanisms. These results warrant further evaluation in the clinical ILP setting and, importantly, in organ perfusion. [J Natl Cancer Inst 2004;96:000-000] [J Natl Cancer Inst 2004;96:1603-10]

Isolated limb perfusion (ILP) is a treatment method in which high concentrations of drugs are administered to a limb containing an unresectable tumor that is temporarily isolated from the rest of the body's circulatory system by the use of an extracorporeal perfusion circuit and a tourniquet placed at the root of the limb. ILP with tumor necrosis factor alpha (TNF- α) and mel-

phalan is associated with synergistic antitumor effects against melanoma (1), large soft-tissue sarcomas (2,3), and various other tumors in the clinical setting (4-6). We have previously shown that the basis for the synergy is both a substantial enhancement of tumor-selective melphalan uptake (7) and the complete destruction of the tumor vasculature (2). The enhanced tissue uptake of different cytotoxic agents, when combined with TNF- α , shown in various limb and liver tumor models in our laboratory (7-12), prompted us to investigate a number of vasoactive substances for similar effects.

Histamine (Hi) is an obvious candidate to enhance tissue uptake of cytotoxic agents during ILP. It is an inflammatory mediator that is formed and stored mainly in the granules of mast cells and basophils, but it has also been identified in epidermal cells, gastric mucosa, neurons of the central nervous system, and in cells in regenerating or rapidly growing tissues. Its effect on fine vessels is to cause edema by increasing the flow of lymph and lymph proteins into the extracellular space and also by promoting the formation of gaps between endothelial cells, thus increasing transcapillary vesicular transport (13). The same mechanism that causes edema in fine vessels could potentially be used to increase drug concentrations in tumor tissues.

In this study, we performed ILP in a rat model by using combinations of Hi and melphalan to determine if Hi would increase the effects of melphalan. To determine the *in vivo*

Affiliations of authors: Department of Surgical Oncology, Erasmus MC, Daniel den Hoed Cancer Center, Rotterdam, The Netherlands (FB, SH, ALBS, STVT, AMME, TLMTH); Department of Experimental Oncology, University of Leuven, Leuven, Belgium (EADB); Lab Experimental Oncology (LEO), University of Leuven, Leuven, Belgium and Departments of Chemistry and Oncology, University of Antwerp, Antwerp, Belgium (GG).

Correspondence to: Timo L. M. ten Hagen, PhD, Erasmus MC, Department of Surgical Oncology, Laboratory of Experimental Surgical Oncology, Rm. Ee 0102a, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands (e-mail: t.l.m.tenhagen@erasmusmc.nl).

See "Notes" following "References."

DOI: 10.1093/jnci/djh300

Journal of the National Cancer Institute, Vol. 96, No. 21, © Oxford University Press 2004, all rights reserved.

mechanisms involved, we measured melphalan uptake and performed histologic analysis after treatment. In addition, cultured sarcoma (14) and normal endothelial cells were treated *in vitro* with Hi, melphalan, or a combination of the two, and cytotoxicity, necrosis, apoptosis, and paracellular permeability were assayed.

MATERIALS AND METHODS

ILP

Male inbred Brown Norway rats weighing 250–300 g were obtained from Harlan-CPB (Austerlitz, The Netherlands) and were fed a standard laboratory diet *ad libitum* (Hope Farms, Woerden, The Netherlands). The studies were done in accordance with protocols approved by the Animal Care Committee of the Erasmus University Rotterdam (Rotterdam, The Netherlands).

Small fragments (diameter = 3 mm) of the spontaneous, nonimmunogenic, syngeneic BN-175 sarcoma (14) were inserted subcutaneously in the right hind legs of the rats, as previously described (8). Tumor growth was measured daily with a caliper, and tumor volume was calculated using the formula $0.4(A^2 \times B)$ (where B represents the largest tumor diameter and A is the diameter perpendicular to it). When tumor diameter exceeded 25 mm or at the end of the experiment, rats were anesthetized and killed by cervical dislocation.

The treatment consisted of the experimental ILP previously described (8,11). In brief, 7–10 days after tumor fragments were inserted (when they reached a diameter of 12–15 mm) rats were anesthetized by intraperitoneal ketamine and intramuscular hypnomidate. An incision parallel to the inguinal ligament was made, and the inguinal vessels were cannulated and connected by way of a low-flow roller pump (Watson Marlow, Falmouth, U.K.) to an oxygenated reservoir where drugs were added, in bolus, to the perfusate (total volume = 5 mL). A groin tourniquet was used to occlude collateral vessels, allowing a proper isolation of the limb. The temperature of the limb was maintained at 38 °C using a warm-water blanket.

The perfusate consisted of hemacel alone (sham) (Boehringer Pharma, Amsterdam, The Netherlands), hemacel plus 40 µg of melphalan (Alkeran Wellcome, Beckenham, UK), hemacel plus 40 µg of melphalan and 1000 µg of Hi (kindly provided by Maxim Pharmaceuticals, San Diego, CA), or hemacel plus 1000 µg of Hi.

To evaluate the role of the different Hi receptors in the Hi-based ILP, the Hi receptor blockers promethazine (H_1 -R) (Centrafarm, Etten-Leur, The Netherlands) and famotidine (H_2 -R) (Sigma, Zwijndrecht, The Netherlands) were added to the perfusate (200 and 50 µg/mL, respectively) and allowed to circulate into the limb for 5 minutes before melphalan and Hi were added.

Tumor dimensions were measured every day and used to monitor tumor volume. Volume on day 9 was compared with that on day 0, and response was classified as follows: progressive disease, increase of more than +25%; no change, volume between –25% and +25%; partial remission, decrease between –25% and –99%; or complete response, no palpable tumor.

Limb function was clinically observed as the ability to walk and stand on the perfused limb after ILP. On a scale from grade 0 to 2, grade 0 is severely impaired function in which the rat

drags its hind limb, grade 1 is slightly impaired function (cannot use it in a normal way but can stand on it), and grade 2 is an intact function (normal walking and standing pattern) (8).

In Vivo Melphalan Uptake

To evaluate melphalan distribution, we killed 11 rats (six treated with Hi plus melphalan and five treated with melphalan alone) immediately after ILP was performed. Tumors and muscle from the limb were removed, snap-frozen in liquid nitrogen, and stored at –80 °C. Tissues were homogenized in 2 mL of acetonitrile with a PRO 200 homogenizer (Pro Scientific, Oxford, CT) and centrifuged at 2500g and 4 °C. Melphalan concentration (reported as nanograms of melphalan per gram of tissue) was measured by gas chromatography–mass spectrometry on at least three different pieces of similar final weight per sample, as described previously (7,15). Given the tumor and muscle values for melphalan uptake, the tumor-to-muscle ratio was calculated, considering the amount of melphalan measured in muscle as 100% and calculating the tumor value in comparison with it.

Histologic Evaluation

Two animals from each treatment group were killed by cervical dislocation directly after ILP; tumors and a piece of muscle from the limb were excised and cut in half. One half was fixed in 4% formaldehyde solution, embedded in paraffin, and stained with hematoxylin and eosin. Images of stained samples were taken on a Leica DM-RXA microscope (Leica Microsystems, Rijswijk, The Netherlands) with a Sony 3CCD DXC camera (Sony Netherlands, Badhoevedorp, The Netherlands).

Cell Culture

BN-175 cells (spontaneous rapidly growing and metastasizing soft-tissue sarcoma) (14) were grown in RPMI 1640 medium (Life Technologies, Leiden, The Netherlands) supplemented with 10% fetal calf serum (FCS) and 0.1% penicillin–streptomycin (Life Technologies). For growth assays, BN-175 cells were plated in 96-well flat-bottomed microtiter plates (Costar, Cambridge, MA) at 10^5 cells per well (in 100 µL) 24 hours before treatment and allowed to grow to confluence. Next, the cells were incubated at 37 °C in 5% CO₂ for 72 hours in the presence of medium alone or medium plus various concentrations of melphalan and Hi. Hi concentrations ranged from 0 to 200 µg/mL. Melphalan concentration ranged from 0 to 8 µg/mL.

HUVECs were prepared by collagenase treatment of freshly obtained human umbilical veins and cultured in human endothelial serum-free medium–RPMI medium (Cambrex Bioscience, Verviers, Belgium) supplemented with 10% heat inactivated human serum (Invitrogen Life Technologies, Breda, The Netherlands), 20% FCS, human epidermal growth factor, human basic fibroblast growth factor, and 0.1% penicillin–streptomycin (Life Technologies). For growth assays, HUVECs were plated 24 hours before treatment at 6×10^4 cells per well and cultured for 48 hours with various concentrations of Hi (0 to 200 µg/mL) and melphalan (0 to 200 µg/mL).

Cell Growth

Growth of BN-175 cells and HUVECs was measured using the Sulforhodamine-B (SRB) assay (16). In brief, cells were

washed with phosphate-buffered saline, incubated with 10% trichloroacetic acid for 1 hour at 4 °C, and washed again with phosphate-buffered saline. Cells were stained with SRB (0.5% SRB in 1% acetic acid) for 15 to 30 minutes, washed with 1% acetic acid, and air-dried. Protein-bound SRB was dissolved in Tris base (10 mM, pH 9.4). Absorbance at 540 nm was measured for each well, and tumor cell growth was calculated according to the following formula: percentage of tumor cell growth = (absorbance of test well/absorbance of control well) × 100%. The Hi concentration leading to 50% reduction in absorbance compared with control (i.e., 50% inhibitory concentration [IC₅₀]) was determined from the growth curve. Each experiment was performed four times in duplicate. The mean of all values and the 95% confidence intervals (CIs) were determined and reported.

HUVEC Morphology and Necrosis-Apoptosis Assays

HUVECs were plated 24 hours before treatment at 6×10^4 cells per well in flat-bottomed 12-well plates (Costar) in a volume of 900 μ L per well and grown to confluence. Cells were then incubated at 37 °C in 5% CO₂ with various concentrations of Hi for various times. At each time point, medium was discarded and replaced with 500 μ L of HUVEC medium plus 0.05% YO-PRO for detection of apoptotic cells (Molecular Probes) or with propidium iodide to detect necrotic cells (Sigma). Cells were incubated for 30 minutes in the dark at 37 °C, and pictures were taken with a Zeiss AxioVert 100M inverted microscope with an AxioCam camera (Carl Zeiss, Sliedrecht, The Netherlands).

Cells were cultured and treated using the time points above with the Vybrant Apoptosis assay kit #3 (Molecular Probes) for both adherent and detached cells. In brief, cells were treated with various concentrations of Hi alone (0 to 200 μ g/ml), melphalan alone (0 or 8 μ g/ml), or with combinations of the drugs for 15 or 30 minutes. Culture medium containing floating cells was removed from the wells and transferred to 5-mL tubes. Adherent cells were washed with RPMI medium, trypsinized with 300 μ L of trypsin-EDTA (Biowhitaker), neutralized with 100 μ L of HUVEC medium containing 20% FCS, and added to the 5-mL tubes. Tubes were centrifuged for 5 minutes at 250g, and the supernatant was discarded. Cells were then incubated in 200 μ L of annexin binding buffer and propidium iodide, with or without annexin V (both reagents from the Vybrant Apoptosis assay kit) at room temperature for 15 minutes in the dark and evaluated by flow cytometry with a FACScan (Becton Dickinson, Alphen aan den Rijn, The Netherlands) flow cytometer. Data were processed with Winmidi software version 2.7 (J. Trotter, Salk Institute, San Diego, CA). Experiments were done three times in duplicate, and the mean and 95% CIs of the percentage of living, apoptotic, and necrotic cells were reported.

Endothelial Cell Monolayer Permeability Assay

HUVECs were plated 48 hours before treatment at 6×10^4 cells per well in a monolayer on a fibronectin-coated polycarbonate membrane (diameter = 6.5 mm; pore size = 0.4 μ m) in a transwell device (Costar). HUVEC medium (1 mL) was added to the lower compartment. Approximately 6 hours after the cells reached confluence, medium in the upper chamber was replaced with 50 μ L of fluorescein isothiocyanate-bovine serum albumin (FITC-BSA) (1 mg/mL; Sigma)

plus 250 μ L of HUVEC medium containing various concentrations of Hi. At the same time, medium in the lower chamber was replaced with 700 μ L of HUVEC medium. Fifty-microliter samples were taken from the lower chamber at various times, and FITC fluorescence was measured with a fluorescence photometer (Victor² FSR; Perkin Elmer, Bucks, U.K.) at 490 nm excitation and 530 nm emission. Values were compared with a standard curve based on known concentrations of FITC-BSA.

Next, to evaluate whether melphalan would have any effect on endothelial cell permeability, directly or in conjunction with Hi, the HUVEC monolayer was exposed to 250 μ L of HUVEC medium alone (control), melphalan at 8 μ g/mL, or Hi at 100 μ g/mL with or without melphalan (8 μ g/mL). Permeability was assayed as described above. Experiments were done three times in duplicate. The data were reported as the mean and 95% CIs of all values.

Statistical Analysis

Tumor growth curves were plotted as means and 95% CIs of the data from all animals. We used repeated-measures analyses of variance on the three most representative days, taken from the growth curve patterns 4, 8, and 10 using SAS Software release 8.2 for Windows 2000 (SAS Institute, Cary, NC) using PROC MIXED. Main effects of treatment and day (three levels: days 4, 8, and 10) were included in the models, as was the interaction between treatment and day. For days in which response was statistically significant, interaction terms were further investigated by testing for differences following treatment on that day.

The data from HUVEC monolayer permeability assays was also analyzed as described above. The effects of treatment and time (5 levels: 0, 15, 30, 45, and 60 minutes) were evaluated.

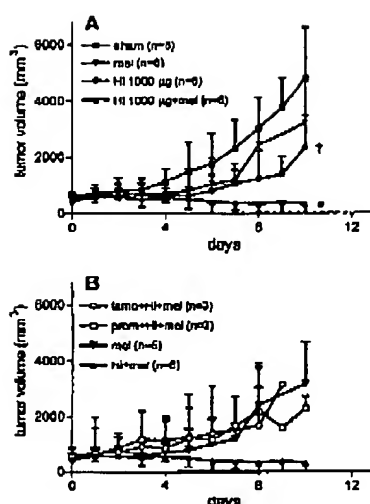
Viability of HUVECs after Hi incubation data was presented and analyzed using the Kruskal-Wallis test with SPSS version 10.0 for Windows 2000.

Melphalan accumulation was shown both as mean values (with 95% CIs) of three measurements performed using different tumor areas and as a ratio between tumor and muscle values, expressed in percentages of tumor versus muscle melphalan uptake. Data were analyzed using the Mann-Whitney *U* test with SPSS version 10.0 for Windows 2000.

Synergism between Hi and melphalan was evaluated by determining whether tumor response after Hi alone or melphalan alone added together was different from the tumor response after Hi plus melphalan. First, the tumor response index was calculated by dividing the initial tumor volume by the tumor volume on a given day after treatment; then, the tumor response index of a rat from the Hi-treated group was randomly added to the tumor response index of a rat from the melphalan-treated group and compared with the tumor response index from the Hi-plus-melphalan group. Next, the data were analyzed with the Mann-Whitney *U* test (exact significance [2 × (one-tailed significance)] using SPSS version 10.0 for Windows 2000.

All statistical tests were two-sided. For all statistical tests, a *P* value less than .05 was considered statistically significant.

Fig. 1. A) Tumor response after histamine-based isolated limb perfusion (ILP): small fragments of BN-175 soft-tissue sarcoma were inserted in the right hind limb of Brown Norway rats (see "Materials and Methods"). After 7 to 10 days when tumors reached 12 to 15 mm in diameter, they were randomly submitted to ILP with perfusate alone (sham), 8 $\mu\text{g/mL}$ melphalan (mel), 200 $\mu\text{g/mL}$ histamine (Hi), or 200 $\mu\text{g/mL}$ Hi plus 8 $\mu\text{g/mL}$ melphalan. Tumors were measured daily with a caliper, and tumor volumes were calculated. When tumor diameter exceeded 25 mm, rats were killed. *, $P < .001$ on day 8 and 10 compared with sham; †, $P = .003$ on day 8 and $P < .001$ on day 10 compared with sham (repeated-measures analyses of variance; two-sided). **B)** Involvement of histamine receptors in histamine-based ILP. Promethazine (H_1 receptor inhibitor, 200 $\mu\text{g/mL}$) or famotidine (H_2 receptor inhibitor, 50 $\mu\text{g/mL}$) were added in bolus to the perfusate and allowed to circulate for 5 minutes before Hi and melphalan were added. Mean tumor volumes and upper 95% confidence intervals are depicted in both graphs. The number of independent experiments (rats) for each treatment is shown in parentheses.



RESULTS

Tumor Response After Hi-based ILP

We previously showed that TNF- α improves the response to ILP by increasing the amount of melphalan delivered to tumor tissues (7). In this study, we used a similar model to test whether another vasoactive molecule, Hi, could also enhance melphalan uptake. A range of Hi concentrations were tested (20 to 200 $\mu\text{g/mL}$), and the concentration that led to optimal tumor regression was determined to be 200 $\mu\text{g/mL}$. Tumors grew exponentially in the Brown Norway rats after control ILP. However, the response to Hi plus melphalan ILP was striking, with a regression (more than a 25% decrease in tumor volume) in four (66%) of the six treated animals, including two (33%) with no palpable tumors approximately 10 days after treatment ($P < .001$). Perfusion with Hi or melphalan alone reduced or stabilized tumor growth—three stable (50%) and one regression (17%) (Fig. 1, A

Table 1. Tumor response after histamine-based ILP*

Treatment	CR (%)	PR (%)	NC (%)	PD (%)
Sham (n = 5)	—	—	—	100
Melphalan (n = 6)	—	17	17	66
Hi (n = 6)	—	—	50	50
Hi + melphalan (n = 6)	33	33	33	—

*ILP = isolated limb perfusion. Volume on day 9 was compared with that on day 0, and response was classified as follows: PD = progressive disease, increase of more than 25%; NC = no change, volume between -25% and 25%; PR = partial remission, decrease between -25% and -99%; CR = complete response, no palpable tumor. Values are expressed in percentage of animals per response per group. — = none.

and Table 1). The combination of Hi plus melphalan showed a synergistic effect because the response index of the combination group was statistically significantly greater than that when the response index from the Hi and melphalan alone groups was randomly added ($P = .043$, Mann-Whitney U test (exact significance [$2 \times$ (one-tailed significance)]).

Perfusion with Hi, either alone or combined with melphalan, did not cause systemic toxicity. Only a transient, mild edema after Hi ILP, both with and without melphalan, was observed, leading to a temporary grade 1 toxicity in two rats for each group. After 2 days, the edema disappeared and limb function returned to normal.

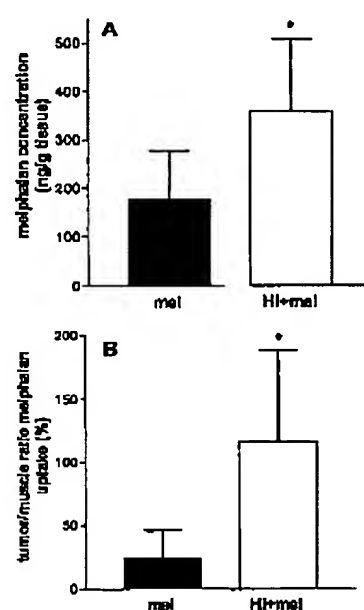
Involvement of Hi Receptors in Hi-based ILP

To determine which Hi receptor (H_1 -R or H_2 -R) is involved in the effects observed above, specific Hi inhibitors were used during the treatment. Both pyrilamine, an H_1 -R blocker, and famotidine, an H_2 -R blocker, could block the effect of Hi in the ILP setting, which means that either H_1 or H_2 receptors are involved (Fig. 1, B).

Indirect Effect of Hi on Tumor Melphalan Uptake

We next evaluated whether Hi treatment could indirectly affect tumor-associated vasculature by increasing vascular permeability, which could cause more melphalan to accumulate in tumors than in normal tissue, as we previously showed using TNF- α combined with melphalan in ILP (7). To compare melphalan uptake in tumors and adjacent muscle, we excised tumors and muscle immediately after ILP with melphalan alone or melphalan combined with Hi and measured melphalan concentration. Hi addition not only led to a twofold increase in the amount of melphalan in tumor tissue ($P = .024$) but also reduced melphalan concentration in the muscle. As a result, adding Hi increased the ratio of melphalan in the tumor to that in the adjacent muscle by four ($P = .02$) (Fig. 2).

Fig. 2. Indirect effect of Hi on the accumulation of melphalan in tumors treated with isolated limb perfusion (ILP). Both tumor and adjacent muscle were excised immediately after ILP and snap-frozen in liquid nitrogen. Melphalan was measured by gas chromatography-mass spectrometry on at least three different pieces per sample as described in "Materials and Methods." **A)** Tumor melphalan concentration. Closed bar, melphalan alone; open bar, Hi plus melphalan. *, $P = .024$ (Mann-Whitney U test, two-tailed). **B)** Ratio between tumor and muscle melphalan uptake. Closed bar, melphalan; open bar, Hi plus melphalan. *, $P = .02$ (Mann-Whitney U test, two-tailed). Mean values with upper 95% confidence intervals are shown.



Histology

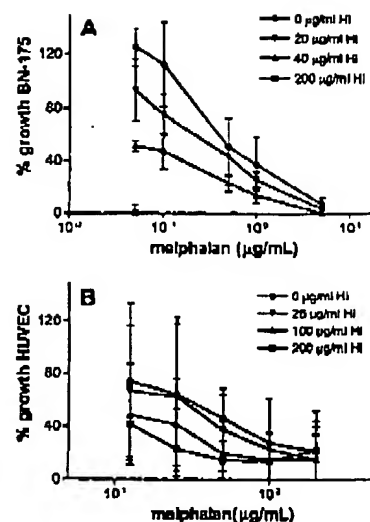
To evaluate both the direct and indirect effects of Hi-based ILP on the tumor and the tumor-associated vasculature, we histologically examined tissue sections taken right after ILP was performed. After ILP with 200 $\mu\text{g/mL}$ Hi alone, scattered vascular damage was observed (Fig. 3). After ILP with 200 $\mu\text{g/L}$ Hi and 8 $\mu\text{g/L}$ melphalan, vascular damage became more pronounced. Perfusion with Hi alone resulted in vasodilatation of the tumor vasculature, extravasation of red blood cells into the tumor, and damage to the endothelial cell lining of tumor vessels. After ILP with Hi and melphalan, most of the tumor vessels were severely damaged and massive hemorrhage was observed. Tumor vessels showed loss of integrity and extensive gap formation, indicating edema. Red and white blood cells observed in the tissue suggested extravasation. We hypothesize that the edema observed in tumor tissue may indicate an augmented influx of melphalan from the blood stream into the tumor. In the muscle, however, no apparent changes in terms of hemorrhage, vasodilatation, or infiltrates after treatment, as above, were observed (data not shown).

These vascular effects were not observed when rats received sham ILP or melphalan via ILP (Fig. 3). After sham ILP, vessels were intact and tumor tissue was unaffected. When tumors were perfused with melphalan alone, some necrosis of the tumor tissue could be observed, but no vascular damage was seen. Together, these results indicate that Hi has tumor vascular-selective activity against the endothelial lining. This vascular effect was even more pronounced when Hi was combined with melphalan.

Cytotoxicity of Hi

The direct cytotoxic effects of Hi on BN-175 tumor cells and HUVEC endothelial cells were evaluated by means of *in vitro* cytotoxicity assays. Cell growth was inhibited in a concentration-dependent manner for both cell lines evaluated. BN-175 tumor cells were more sensitive to Hi, with an IC_{50} of 30 $\mu\text{g/mL}$. HUVEC appeared less sensitive to Hi with an IC_{50} of approximately 100 $\mu\text{g/mL}$ (Fig. 4). The cytotoxic effect of Hi combined with melphalan *in vitro* was not synergistic, it was only additive.

Fig. 4. *In vitro* cytotoxicity of Hi according to percentage of tumor growth inhibition. Cells were incubated for 72 hours with different concentrations of Hi with or without melphalan, and cell growth was evaluated using the Sulphorhodamine B assay as described in "Materials and Methods." A) BN-175 sarcoma (Hi IC_{50} inhibitory concentration [IC_{50}] of 30 $\mu\text{g/mL}$); B) Human umbilical vascular endothelial cells (HUVECs) (Hi IC_{50} of 100 $\mu\text{g/mL}$). Each point represents an average of four independent experiments. Error bars show 95% confidence intervals of the mean.



Direct Effect on HUVEC: Morphology and Apoptosis Assay

In vitro, Hi was only slightly cytotoxic to HUVEC cells after long-term treatment (Fig. 4, B). Moreover, addition of Hi to melphalan did not enhance the sensitivity of HUVEC toward melphalan (Fig. 4, B). However, after ILP, a strong effect of Hi on the endothelial lining of tumor vessels was observed (Fig. 3). Therefore, we examined the morphology of HUVECs after short incubations (no longer than 60 minutes) with Hi plus melphalan. We observed a dose- and time-dependent effect of Hi on HUVEC, starting with the appearance of gaps between the cells. As time progressed, some cells became rounded and others became extended. In the higher concentration range or after prolonged incubation, cell fragments were seen in the medium (Fig. 5). Cells exposed to medium alone did not show these morphologic changes.

The observed differences in HUVEC morphology after Hi treatment prompted us to investigate whether these changes

Fig. 3. Histology of tumor and adjacent muscle after isolated limb perfusion (ILP). Tumors and muscle were excised immediately after and 24 hours after ILP for each treatment, fixed in 4% formaldehyde solution, and embedded in paraffin for hematoxylin-eosin staining. Perfusate alone (sham) ILP with intact vessels and normal tumor tissue; melphalan 8 $\mu\text{g/mL}$ ILP with some spots of necrosis on tumor tissue but no vascular damage; Hi-alone 200 $\mu\text{g/mL}$ ILP showing vascular vasodilatation, extravasation of red blood cells into the tumor and damage to the endothelial cell lining of tumor vessels; Hi plus melphalan (200 $\mu\text{g/mL}$ and 8 $\mu\text{g/mL}$, respectively) ILP showing the damage to tumor vessels and massive hemorrhage. Pictures illustrate representative examples of each treatment.

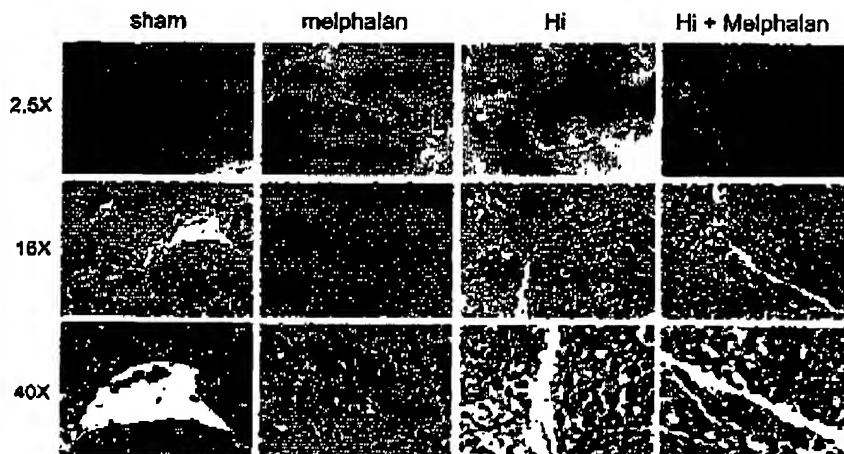
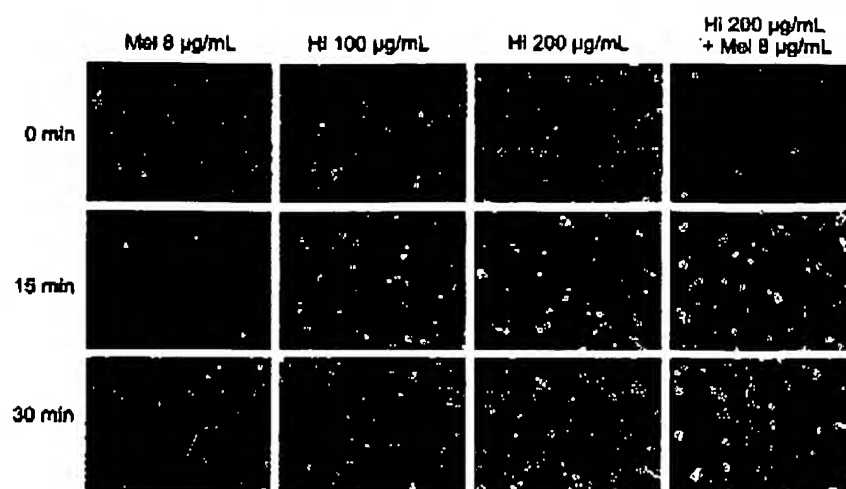


Fig. 5. Direct effect of Hi on endothelial cells. Human umbilical vascular endothelial cells (HUVECs) were incubated with medium alone, melphalan (8 $\mu\text{g/mL}$) alone, Hi (100 $\mu\text{g/mL}$ or 200 $\mu\text{g/mL}$) alone or in combination for 15 and 30 minutes. Gap formation and morphologic changes can be observed already after 15 minutes incubation both with 100 and 200 $\mu\text{g/mL}$ (a more pronounced effect for 200 $\mu\text{g/mL}$)

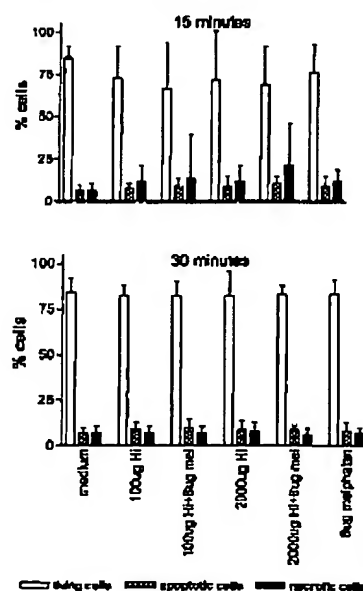


were irreversible, that is, whether they could lead to apoptosis or necrosis. With YO-PRO and propidium iodide to detect apoptosis and necrosis of adherent cells, respectively, we found no differences in the number of apoptotic or necrotic cells after exposure of HUVECs to Hi compared with exposure to medium alone (data not shown). When all cells, adherent as well as detached, were examined using the Vybrant apoptosis assay, no increase in the number of apoptotic cells or the number of necrotic cells was observed when Hi was added compared with medium alone ($P = .4$ and $P = .5$, respectively) (Fig. 6). Moreover, when Hi was combined with melphalan, still no increase in the number of apoptotic or necrotic cells was seen.

Hi and Paracellular Permeability *In Vitro*

We observed an increase in melphalan concentration in tumors treated with both drugs, which was accompanied by strong

Fig. 6. Viability of human umbilical vascular endothelial cells (HUVECs) after short incubation with Hi. HUVECs were plated 24 hours before treatment at 6×10^4 cells per well and grown to confluence. Cells were cultured at 37 °C in 5% CO_2 with different concentrations of Hi (0 to 200 $\mu\text{g/mL}$) and melphalan (0 or 8 $\mu\text{g/mL}$) for 15 or 30 minutes. The Vybrant Apoptosis assay kit was used to detect apoptosis and necrosis of adherent and detached cells prior to flow cytometric evaluation of the cells. Experiments were done three times in duplicate. The mean percentage, per group of cells, and upper 95% confidence intervals are shown.



effect of Hi on the tumor associated vasculature after ILP (Figs. 2 and 3). Histopathologic examination revealed the Hi-induced formation of gaps *in vivo* in a concentration-dependent manner, requiring a minimum concentration of 200 $\mu\text{g/mL}$ (data not shown). Andriopoulou et al. (17) reported that incubation of microvascular endothelial cells for 25 minutes with a relatively low Hi concentration (11 $\mu\text{g/mL}$) resulted in a 120% and 45% increase in permeability for long- and recently confluent cultures, respectively. We investigated the pattern of permeability using Hi concentrations 10-fold higher than in that study. In line with the findings of Andriopoulou et al. (17), we found a concentration- and time-related effect of Hi on HUVEC monolayer permeability as well as a sharper increase in permeability in the first 15 minutes. The results presented in Fig. 7. A show that exposure of HUVEC to 200 $\mu\text{g/mL}$ Hi alone resulted in an increase in permeability of fivefold (5.6, 95% CI = 3.5 to 7.7) compared with the control, and 100 $\mu\text{g/mL}$ Hi alone resulted in a two- to threefold (2.8, 95% CI = 1.5 to 4.1) increase compared with the control. Incubation with 50 $\mu\text{g/mL}$ Hi caused only a very slight increase of about 1.5-fold (1.5, 95% CI = 1.0 to 2.0). Interestingly, when HUVECs were exposed to 50 $\mu\text{g/mL}$ or 100 $\mu\text{g/mL}$ Hi, no additional effect on permeability was observed after 15 and 30 minutes of incubation (curves start to parallel the control), respectively. Exposure of HUVECs to 200 $\mu\text{g/mL}$ Hi resulted in an ongoing response of HUVECs as shown by the continuing permeability increase compared with control. Even at 60 minutes, the response of HUVECs to Hi did not parallel the control curve. Incubation with melphalan had no effect on the permeability of HUVEC monolayer, neither alone nor in combination with Hi (Fig. 7, B). The ongoing permeability increase might be essential to the observations *in vivo*.

DISCUSSION

This study shows for the first time, to our knowledge, the activity of Hi plus melphalan in ILP for the treatment of soft-tissue sarcomas. The strong effect of Hi-based ILP with melphalan was explained by three mechanisms: 1) direct cytotoxicity to the tumor cells, 2) direct cytotoxicity to the tumor-associated vasculature, and 3) an indirect effect through Hi-mediated, increased melphalan concentration in the tumor.

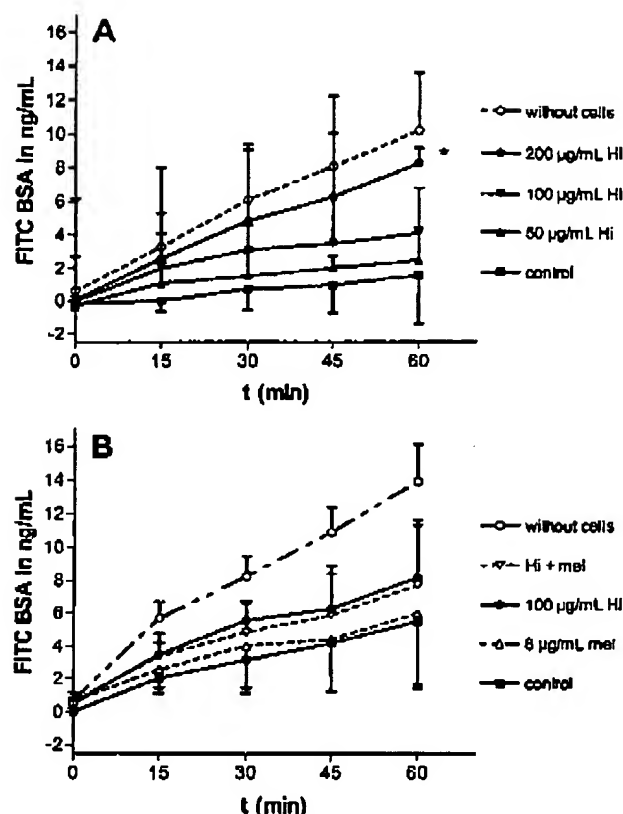


Fig. 7. Effect of Hi on human umbilical vascular endothelial cell (HUVEC) monolayer permeability. HUVECs were cultured on the filter of a transwell unit for 48 hours before the addition of fluorescein isothiocyanate and bovine serum albumin (FITC-BSA)-containing medium (control) or A) Hi in different concentrations, B) plus or minus melphalan to the upper compartment (see "Material and Methods"). The amount of FITC-BSA in the lower compartment was measured every 15 minutes for an hour. Values are from three experiments, each done in duplicate. Error bars show 95% confidence intervals of the mean. *, *P* values using repeated-measures analysis of variance test *P* = .001 for 200 µg/mL Hi at 15, 30, 45, and 60 minutes compared with control.

The direct inhibitory effect of Hi on tumor cells is in accordance with previous reports on Hi receptor expression on different cell lines and human neoplasias, suggesting that it might regulate tumor cell growth (18, 19). This growth-inhibitory effect on the tumor cells, combined with the observed direct effect on the endothelial cells, seen by us both *in vitro* and *in vivo*, might be an explanation for the antitumor effect of Hi alone (50% of the tumors stopped growing), compared with control perfusions (all tumors continued to grow). Nevertheless, chemotherapeutic drugs, such as melphalan, for example, must be added to the ILP to achieve a good antitumor response, which coincides with our observations in TNF- α -based ILP (8).

The direct effect of Hi on endothelial cells *in vitro* is more pronounced than that of TNF- α , the current drug of choice for ILP, which we believe adds to the observed tumor response *in vivo*. Hi alone is capable of changing the morphology of endothelial cells after a short incubation period, resulting in gap formation and rounded cells, as shown in Fig. 5. When combined with melphalan *in vivo*, the effect on the vasculature is

much more evident, with diffuse gap formation and destruction of endothelial cell lining observed immediately after the ILP. In the standard treatment using TNF- α plus melphalan, destruction of the endothelial lining is a secondary effect and takes a couple of days to become evident (20). Therefore, ILP with Hi would likely enhance drug uptake more quickly and effectively than ILP with TNF- α .

The *in vitro* permeability results were in accordance with the *in vivo* findings of an augmented uptake of melphalan in the tumor as well as a decrease in the muscular concentration, reducing regional toxicity. It is remarkable that the Hi concentration used in the ILP (200 µg/mL) led to a continuous increase in the permeability of endothelial cells, which is different from the standard described short-term effect of Hi that occurs only for the first 15 minutes of exposure (18). We speculate that with the Hi concentration used in the ILP, a threshold is reached that triggers a prolonged cellular response, a supposition that is currently under investigation.

Another potential advantage of Hi over TNF- α is its pharmacokinetics. Hi has a very short half-life in serum—0.35 minutes versus 20 minutes for TNF- α (21). Hi is metabolized through two major pathways in humans: the main pathway involves ring methylation and is catalyzed by the enzyme histamine-*N*-methyltransferase, which is widely distributed in the tissues. Most of the product, *N*-methylhistamine, is converted by monoamine oxidase to *N*-methylimidazole acetic acid. Alternatively, Hi can undergo oxidative deamination, catalyzed mainly by the nonspecific enzyme diamine oxidase. The products are imidazole acetic acid and its riboside, which have little or no activity and are excreted in the urine (13). Although these data come from studies with lower dosages or endogenous Hi, the wide distribution of and fast action of the enzymes that metabolize Hi means that Hi is a potentially safer drug than TNF- α in case of leakage into the systemic circulation during ILP. Furthermore, these properties of Hi pharmacokinetics open new possibilities of application in, for example, isolated liver perfusion. More studies on the pharmacokinetics of higher doses and evaluation in the clinical setting are, however, essential for the clinical translation of Hi.

Our findings support a tumor endothelial cell-specific targeting effect of Hi resulting in dramatic hemorrhage and destruction of the endothelial cell lining of tumor vessels (confirmed with CD-31 staining [data not shown]) *in vivo*. We hypothesize that the pronounced direct effect of Hi on the endothelial cell lining is fundamental for the better response than that achieved by melphalan alone in the ILP model discussed here.

H₁ and H₂ Hi receptors were involved in Hi-induced tumor regression in our model. Each receptor inhibitor alone blocked the Hi effect *in vivo*. The two receptors are located in different cell types and have independent mechanisms of action: H₁ has a higher affinity, a rapid but short-lived effect, and is located in the endothelial cells; H₂ has a lower affinity, a slower but more sustained effect, and is located in the vascular smooth muscle cells.

Toxicity would be unlikely to be a limiting factor for the use of Hi in ILP in humans because no systemic toxicity was observed, and the regional toxicity, affecting 33% of the rats receiving Hi either alone or combined with melphalan, was very mild and completely reversible after 2 days of recovery. Accordingly, ILP with TNF and melphalan in the clinical setting, as Hi plus melphalan did in the animal model, also results in

erythema and edema, which sometimes slightly impairs motility (grades II and III of Wieberdink, respectively) in most of the patients (6,22)

In conclusion, Hi combined with melphalan had a striking effect in the ILP for the treatment of soft-tissue sarcomas in rats. The mechanism of action involved both direct and indirect effects—cytotoxicity on the tumor and endothelial cells and tumor-associated vasculature with a twofold increase in the tumoral uptake of melphalan combined with a reduction in the uptake in the adjacent muscle. Therefore, Hi plus melphalan in ILP seems to be a promising alternative to TNF- α , to be evaluated in the clinical setting.

REFERENCES

- (1) Lienard D, Lejeune FJ, Ewalenko P. In transit metastases of malignant melanoma treated by high dose rTNF alpha in combination with interferon-gamma and melphalan in isolation perfusion. *World J Surg* 1992;16:234-40.
- (2) Eggermont AM, Schraffordt KH, Lienard D, Kroon BB, van Geel AN, Hoekstra HJ, et al. Isolated limb perfusion with high-dose tumor necrosis factor-alpha in combination with interferon-gamma and melphalan for nonresectable extremity soft tissue sarcomas: a multicenter trial. *J Clin Oncol* 1996;14:2653-65.
- (3) Eggermont AM, Schraffordt KH, Klausner JM, Kroon BB, Schlag PM, Lienard D, et al. Isolated limb perfusion with tumor necrosis factor and melphalan for limb salvage in 186 patients with locally advanced soft tissue extremity sarcomas. The cumulative multicenter European experience. *Ann Surg* 1996;224:756-64.
- (4) Olieman AP, Lienard D, Eggermont AM, Kroon BB, Lejeune FJ, Hoekstra HJ, et al. Hyperthermic isolated limb perfusion with tumor necrosis factor alpha, interferon gamma, and melphalan for locally advanced nonmelanoma skin tumors of the extremities: a multicenter study. *Arch Surg* 1999;134:303-7.
- (5) Bickels J, Manusama ER, Gutman M, Eggermont AM, Kollender Y, Abu-Abid S, et al. Isolated limb perfusion with tumor necrosis factor-alpha and melphalan for unresectable bone sarcomas of the lower extremity. *Eur J Surg Oncol* 1999;25:509-14.
- (6) Eggermont AM, de Wilt JH, ten Hagen TL. Current uses of isolated limb perfusion in the clinic and a model system for new strategies. *Lancet Oncol* 2003;4:429-37.
- (7) de Wilt JH, ten Hagen TL, de Bock G, van Tiel ST, de Bruijn EA, Eggermont AM. Tumor necrosis factor alpha increases melphalan concentration in tumor tissue after isolated limb perfusion. *Br J Cancer* 2000;82:1000-3.
- (8) de Wilt JH, Manusama ER, van Tiel ST, van Ijken MG, ten Hagen TL, Eggermont AM. Prerequisites for effective isolated limb perfusion using tumor necrosis factor alpha and melphalan in rats. *Br J Cancer* 1999;80:161-6.
- (9) Manusama ER, Nooljen PT, Stavast J, Duran NM, Marquet RL, Eggermont AM. Synergistic antitumor effect of recombinant human tumor necrosis factor alpha with melphalan in isolated limb perfusion in the rat. *Br J Surg* 1996;83:551-5.
- (10) Manusama ER, Stavast J, Duran NM, Marquet RL, Eggermont AM. Isolated limb perfusion with TNF alpha and melphalan in a rat osteosarcoma model: a new anti-tumour approach. *Eur J Surg Oncol* 1996;22:152-7.
- (11) van der Veen AH, de Wilt JH, Eggermont AM, van Tiel ST, Scynhaeve AL, ten Hagen TL. TNF-alpha augments intratumoural concentrations of doxorubicin in TNF-alpha-based isolated limb perfusion in rat sarcoma models and enhances anti-tumour effects. *Br J Cancer* 2000;82:973-80.
- (12) van Euten B, de Vries MR, van Ijken MG, Lans TE, Gueters G, Ambagtsheer G, et al. Degree of tumour vascularity correlates with drug accumulation and tumour response upon TNF-alpha-based isolated hepatic perfusion. *Br J Cancer* 2003;88:314-19.
- (13) Garrison JC. Histamine, bradykinin, 5-hydroxytryptamine and their antagonists. In: Alfred Goodman Gilman, Theodore W. Rall, Alan S. Nies, Palmer Taylor, editors. *The pharmacological basis of therapeutics*. 8th ed. Elmsford (NY): Pergamon Press, 1990:575-99.
- (14) Kon WJ, Zondervan PE, Hulsman LO, Weijma IM, Westbroek DL. Incidence of spontaneous tumors in a group of retired breeder female brown Norway rats. *J Natl Cancer Inst* 1984;72:709-13.
- (15) de Bock G, van Cauwenbergh K, Eggermont AM, van Oosterom AT, de Bruijn EA. Determination of melphalan and hydrolysis products in body fluids by GC-MS. *J High Resolut Chromatogr* 1997;20:697-700.
- (16) Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, et al. New colorimetric cytotoxicity assay for anticancer-drug screening. *J Natl Cancer Inst* 1990;82:1107-12.
- (17) Andriopoulou P, Navarro P, Zanetti A, Lampugnani MG, Dejana E. Histamine induces tyrosine phosphorylation of endothelial cell-to-cell adhesion junctions. *Arterioscler Thromb Vasc Biol* 1999;19:2286-97.
- (18) Cricco G, Martin G, Labombarda P, Cocco C, Bergoc R, Rivera E. Human pancreatic carcinoma cell line Pano-1 and the role of histamine in growth regulation. *Inflamm Res* 2000;49 Suppl 1:S68-9.
- (19) Valencia S, Hernandez-Angeles A, Soria-Jasso LE, Arias-Montano JA. Histamine H(1) receptor activation inhibits the proliferation of human prostatic adenocarcinoma DU-145 cells. *Prostate* 2001;48:179-87.
- (20) Nooljen PT, Manusama ER, Eggermont AM, Schalkwijk L, Stavast J, Marquet RL, et al. Synergistic effects of TNF-alpha and melphalan in an isolated limb perfusion model of rat sarcoma: a histopathological, immunohistochemical and electron microscopical study. *Br J Cancer* 1996;74:1908-15.
- (21) Rizell M, Naredi P, Lindner P, Hellstrand K, Samo M, Jansson PA. Histamine pharmacokinetics in tumor and host tissues after bolus-dose administration in the rat. *Life Sci* 2002;70:969-76.
- (22) Wieberdink J, Benckhuijsen C, Braas RP, van Slooten EA, Olthuis GA. Dosimetry in isolation perfusion of the limbs by assessment of perfused tissue volume and grading of toxic tissue reactions. *Eur J Cancer Clin Oncol* 1982;18:905-10.

NOTES

F. Brunstein was supported by a grant from CAPES-MEC Brazil, process number 1237/01-2. A. Eggermont was supported by a grant from Maxim Pharmaceuticals.

We thank Maxim Pharmaceuticals, Inc., San Diego, CA, for kindly providing histamine dihydrochloride injections for the studies. We thank Gerard J. J. M. Borsboom from the Department of Public Health, Erasmus MC University Medical Center Rotterdam, Rotterdam, The Netherlands, for expert assistance with statistical evaluation of the manuscript.

Manuscript received March 5, 2004; revised August 30, 2004; accepted August 31, 2004.

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☒ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.